Evaluation of Alternative Host Bacteria as Vehicles for Oral Administration of Bacteriophages

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Abstract: Survival of bacteriophages through the upper gastrointestinal tract (UGIT) and persistence in the lower gastrointestinal tract (LGIT) is essential for treatment of enteric bacterial infections. We have hypothesized that non-pathogenic Alternative Host Bacteria (AHB), originally isolated from poultry cecal samples, could be used to protect bacteriophages during UGIT passage and to provide host cells for continued amplification in the LGIT. We selected two previously-identified Wide Host Range (WHR) bacteriophages (WHR-8 and WHR-10) and their respective AHB for use in the present studies. For each of the bacteriophage-host combinations, combination of the bacteriophage with the AHB prior to oral gavage had little effect on the concentration of recovered bacteriophages from the cecal contents during the three days post-administration. Furthermore, continuous administration of the AHB in the drinking water had little effect on intestinal bacteriophage recovery during the three days of evaluation. Bacteriophages were also tested for differences in anaerobic and aerobic lysis of *Salmonella enteritidis* as a possible reason for decreased persistence in the LGIT. Differences in lysis between anaerobic and aerobic environments were significant, however levels were not likely different enough to have significant *in vitro* effects. These results suggest that selection of AHB to protect or amplify enteric bacteriophage populations is not necessarily a simple process. Survival of the AHB and ability of the AHB to replicate in the LGIT of the target animals are among considerations that should be made in future investigations.

Key words: Bacteriophages, *Salmonella*, alternative host, bacteria, chickens

Introduction

Bacteriophages have been used with some success at eliminating poultry pathogens (Barrow *et al.*, 1998; Huff *et al.*, 2003a), although treatment of enteric bacterial infections has been problematic (Berchieri *et al.*, 1991). For therapeutic enteric (oral) administration, the initial low pH of the upper gastrointestinal tract (UGIT) has been shown to be highly detrimental to bacteriophage survival and arrival at the lower gastrointestinal tract (LGIT) where many infections, such as *Salmonella*, are most prominent. Higgins (2002) subjected *Salmonella enteritidis* (SE) bacteriophages to low pH similar to that of the UGIT and recovered very few bacteriophages, suggesting that bacteriophages are unlikely to survive at high enough titers to reach the LGIT, a common site of infection for *Salmonella*, to be effective. Similar results have been shown by other investigators in other species (Smith *et al.*, 1987). Kudva *et al.* (1999) reported that phages effective against *E. coli* O157:H7 in aerobic conditions failed to effectively lyse bacteria under anaerobic conditions, rendering them inappropriate for use in the gastrointestinal tract. However, some reports have shown no difference between anaerobic and aerobic lysis of *E. coli* O157:H7 by bacteriophages (Raya *et al.*, 2006; Tanji *et al.*, 2005). Interestingly, the bacteriophages that reportedly killed *E. coli* O157:H7

under both conditions were effective at reducing intestinal carriage of the pathogen in sheep (Raya *et al.*, 2006) and mice (Tanji *et al.*, 2005). These results suggest that proper selection of therapeutic bacteriophages for the treatment of enteric pathogens should include *in vitro* effectiveness in anaerobic conditions.

Presently, we evaluated the use of non-pathogenic Alternative Host Bacteria (AHB) as a vehicle for the administration of bacteriophages for survival through the UGIT. Continuous delivery of the AHB in the drinking water was also evaluated for potential to serve as an additional amplification host for Wide Host Range (WHR) bacteriophages as these viruses do not typically remain in an environment without host bacteria (Merril *et al.*, 1996). In addition to GIT passage we evaluated bacteriophages selected for their ability to lyse in anaerobic and aerobic conditions.

Materials and Methods

Bacteriophages: Bacteriophages were propagated and enumerated as previously described (Higgins *et al.*, 2005). Two WHR bacteriophages, originally isolated from wastewater against *Salmonella enteritidis*, were previously selected (companion paper published in this number) which could amplify in non-pathogenic AHB

(WHR-8: *E. coli*, WHR-10: *Klebsiella oxytoca*). These bacteria were previously selected for their ability to inhibit *in vitro* growth of SE and were able to reduce SE recovery in poults (Bielke *et al.*, 2003).

Experiment 1: In this experiment, the effect of oral co-administration of WHR with AHB with or without AHB administration in the drinking water was compared for persistence of WHR in the intestinal tract during a three day study. Day-of-hatch chicks (N = 300) were randomly assigned to one of 4 treatment groups: 1) WHR-8+AHB by oral gavage, 2) WHR-8+AHB by oral gavage with AHB in the drinking water, 3) WHR-10+AHB by oral gavage and 4) WHR-10+AHB by oral gavage with AHB in the drinking water.

Bacteriophage WHR-8 at 2×10^8 PFU or WHR-10 at 3×10^8 PFU were allowed to incubate at 37°C for 10 min prior to administration by oral gavage (100 µL) with approximately 10^7 CFU of their respective AHB to allow the bacteriophages to infect the AHB. This time was not likely long enough for bacteriophages to lyse AHB before administration, thus they would be within the bacterial cell during passage through the LGIT. Groups receiving AHB in the drinking water received a 100-fold dilution of fresh overnight culture of their respective AHB in tryptic soy broth[1] (~10^7 cfu/mL) daily in 1% dry skim milk.

At 6 h, 26 h and 77 h post-gavage a composite intestinal sample including ceca, lower small intestine and large intestine were aseptically removed from 25 chicks per group. Samples from 5 birds were pooled in a single sample bag and 5 pools were stomached and evaluated for bacteriophage enumeration. Titer was determined using soft agar overlay plates with SE as previously described (Higgins *et al.*, 2005).

Experiment 2: In this experiment, recovery of enteric WHR-10 was determined at 6, 24, or 72 hours when: 1) WHR-10 alone was administered by gavage; 2) WHR-10 was co-administered with its AHB once by gavage at placement; 3) WHR-10 was co-administered with its AHB by gavage at placement and chicks were continuously exposed to low concentrations; 4) WHR-10 was co-administered with its AHB by gavage at placement and chicks were continuously exposed to high concentrations of its AHB in the drinking water; or 5) WHR-10 and AHB were co-administered by gavage every 12 hours. In all cases, WHR-10 was administered by gavage at 1.5×10^8 PFU/chick alone, or in combination with AHB, in a total gavage volume of 100 µL. The AHB was provided at either a low (5.35 $\log_{10}$/mL) or high (6.35 $\log_{10}$/mL) dose in the drinking water in 1% dry skim milk, prepared fresh daily.

Anaerobic and Aerobic Lysis

Media and culture preparation: Tryptic soy agar[2] plates were prepared normally and stored in the anaerobic chamber[3] for 24 h prior to each experiment. Soft agar tubes were autoclaved and immediately placed into the chamber for cooling.

Statistical analysis: A total of 5 gastrointestinal tracts were pooled for each sample within times and 5 samples were subjected to analysis within each treatment and time point. Data were analyzed within time points using the General Linear Models procedure (GLM) of SAS (SAS Institute, 2002). For lysis studies, a total of 10 soft agar plates were evaluated for each bacteriophage isolate (8 and 10) for analysis of *Salmonella* lysis. Data was also analyzed using the GLM procedure of SAS (SAS Institute, 2002). In both cases, significance was reported at $p < 0.05$.

Anaerobic *vs.* Aerobic Lysis of *Salmonella enteritidis*: SE and bacteriophages were prepared under aerobic conditions according to Higgins *et al.* (2005). For both experiments, soft agar overlay plates were poured with ~10^7 CFU/mL SE and incubated at room temperature (~24 C). Aerobic plates were incubated on the countertop adjacent to the anaerobic chamber. The anaerobic chamber was filled with 85% N_2, 10% H_2 and 5% CO_2 gases. Plaques were counted after overnight incubation.

Results and Discussion

In Experiment 1, a general decline in recoverable WHR-8 and WHR-10 were observed during the course of the study (Fig. 1). While there were subtle differences in phages recovered, there were no significant differences between treatments at any of the times evaluated, even where very low bacteriophage numbers were recovered (WHR-10+AHB only). Overall, there was little indication that provision of the AHB in the drinking water in this experiment caused any increase in bacteriophage recovery. Similarly in Experiment 2, a general decline in WHR-10 bacteriophage recovery occurred over time (Fig. 2). No significant treatment-related differences were observed within times, suggesting that neither co-administration with AHB nor administration of AHB in the drinking water were effective for improving bacteriophage recovery from the intestinal tract.

In addition, these studies showed a significant difference between anaerobic and aerobic lysis of *Salmonella*, however differences were small (Table 1). These data suggest that these particular bacteriophages, selected for *in vivo* lysis of SE, would not likely be inhibited by the anaerobic environment of the LGIT. These results agree with Raya *et al.* (2006) and Tanji *et al.* (2005) that found little difference between anaerobic and aerobic lysis of *E. coli* O157:H7. However, when selecting bacteriophages for enteric treatment, anaerobic and aerobic lysis should be an important selection criterion as Kudva *et al.* (1999) found that
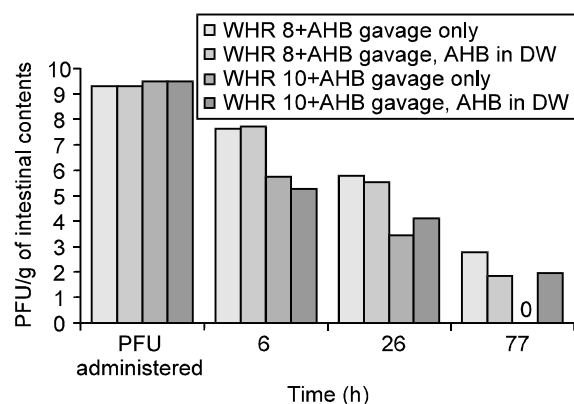
Fig. 1: Recovery of Wide Host Range (WHR) Bacteriophages 8 and 10 from Lower Gastrointestinal Tract of Broiler Chicks. Day of hatch broilers were administered bacteriophages and Alternative Host Bacteria (AHB) by gavage only or with the addition of AHB in the Drinking Water (DW). Lower ileum, cecae and large intestine were combined from 5 chicks per sample and 5 samples were determined for each treatment group at 6, 26 and 77 h post-gavage. PFU were determined using serial dilution and plaque enumeration on soft agar overlay. There were no significant (p>0.05) differences within times

Table 1: Aerobic and anaerobic lysis of *Salmonella enteritidis* by selected bacteriophages

	Phage 8[1]	Phage 10[1]
Aerobic	10.22±0.06[a]	8.83±0.07[a]
Anaerobic[2]	9.75±0.08[b]	9.28±0.04[b]

[1]Bacteriophages prepared in aerobic conditions with *Salmonella enteritidis;* [2]Anaerobic conditions -85% N_2, 10% H_2, 5%CO_2; [a-b]means with different superscripts are significantly (p<0.05) within columns

bacteriophages that successfully killed *E. coli in vitro* had little *in vivo* effect because of decreased activity in anaerobic conditions. While not apparently important for the bacteriophages selected for use in the present study, changes in lytic ability could be due to expression of different genes and proteins by bacteria in anaerobic conditions (Becker *et al.*, 1997; Zhang *et al.*, 1996) (Table 1). Phenotypic changes can be detrimental to the life cycle of a bacteriophage since bacteriophages typically attach to specific expressed proteins, insert their genome at specific points and depend on metabolic processes of the host cell that can change during anaerobiosis.

These studies do not eliminate the possibility of eventual use of AHB for either protection of bacteriophage cocktails or for enteric amplification of desirable bacteriophage populations within the gastrointestinal tract. There are numerous possibilities
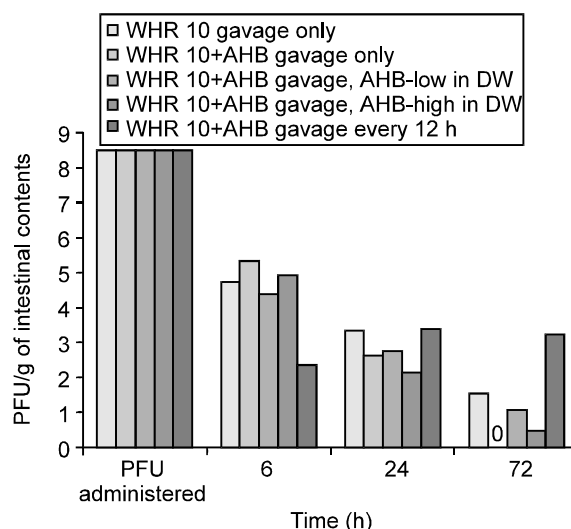

Fig. 2: Recovery of Wide Host Range (WHR) Phage 10 from Lower Gastrointestinal Tract of Broiler Chicks. Phage was administered by oral gavage alone or with Alternative Host Bacteria (AHB) Treatments were WHR 10 gavage only, WHR 10 combined with AHB, or WHR 10+AHB by gavage combined with continuous administration of low (5.35 log_{10}/mL) or high (6.35 log_{10}/mL) concentration of AHB in the Drinking Water (DW). Lower ileum, cecae and large intestine were combined from 5 chicks per sample and 5 samples were determined at 6, 24 and 72 h post-gavage. PFU were determined using serial dilution and plaque enumeration on soft agar overlay. There were no significant (p>0.05) differences within times

for the apparent lack of effect of co-administration of these AHB with their respective WHR bacteriophages including low viability of AHB during UGIT passage or low viability of AHB within the LGIT, providing poor bacteriophage host function. Many possibilities, including these, were not investigated in these preliminary experiments. However, these results do indicate that successful generation of a library of wide host range bacteriophages, which can be protected and amplified *in vivo* using non-pathogenic AHB, may be difficult to achieve.

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